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p97 Disease Mutations Modulate Nucleotide-Induced Conformation to Alter Protein–Protein Interactions

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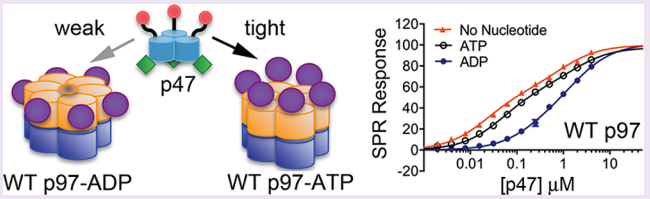
Supporting Information

ABSTRACT: The AAA+ ATPase p97/VCP adopts at least three conformations that depend on the binding of ADP and ATP and alter the orientation of the N-terminal protein–protein interaction (PPI) domain into "up" and "down" conformations. Point mutations that cause multisystem proteinopathy 1 (MSP1) are found at the interface of the N domain and D1-ATPase domain and potentially alter the conformational preferences of p97. Additionally, binding of "adaptor" proteins to the N-domain regulates p97's catalytic activity. We propose that p97/adaptor PPIs are coupled to p97 conformational states. We evaluated the binding of nucleotides and the adaptor proteins p37 and p47 to wild-type p97 and MSP1 mutants. Notably, p47 and p37 bind 8-fold more weakly to the ADP-bound conformation of wild-type p97 compared to the ATP-bound conformation. However, MSP1 mutants lose this nucleotide-induced conformational coupling because they destabilize the ADP-bound, "down" conformation of the N-domain. Loss in conformation coupling to PPIs could contribute to the mechanism of MSP1.



p97/VCP belongs to the AAA+ (ATPase associated with various cellular activities) family of enzymes and is a key regulator of several cellular processes involved in protein homeostasis.[1−3] p97 forms a barrel-like homohexamer with each subunit consisting of tandem ATPase domains (D1 and D2).[4,5] The ATPase domains are sandwiched by N- an C-terminal domains, which function as protein–protein interaction (PPI) sites.[6,7] Binding of a network of adaptor proteins to the terminal domains directs p97 toward a particular cellular process; e.g., binding of the adaptor p47 directs p97 to function in membrane fusion, whereas binding to NPL4/UFD1 targets p97 to ER-associated degradation.[1,8] Recent high-resolution cryo-EM structures of p97 reveal that this enzyme adopts at least three conformations that depend on the binding of ADP and ATPγS in its two ATPase domains.[9] Conformational changes include the orientation of the N-terminal PPI domain, which can adopt an "up" conformation in the presence of ATPγS and a "down" conformation when ADP is bound.[10] However, we still lack mechanistic details about how the nucleotide-induced conformational changes in p97 affect PPIs.

Point mutations in p97 cause the multisystem proteinopathy type 1 (MSP1) disorder, also called inclusion body myopathy associated with Paget's disease and frontotemporal dementia/amyotrophic lateral sclerosis (IBMPFD/ALS).[11,12] MSP1 mutations cluster at the ND1 interface to affect p97's function.[13] Although these mutations do not alter the hexameric state of p97, they cause an overall ∼3-fold increase in p97 D2 ATPase activity[13−15] and decrease the affinity of ADP to the isolated ND1 domains of p97.[10,16] In addition, immunoprecipitation studies found that MSP1 disease mutations caused differential binding of adaptor proteins to the N-domain of p97 in cells.[15,17,18] However, this differential immunoprecipitation was not observed *in vitro* using purified proteins.[19−21] This discrepancy may be resolved by accounting for the ATPase hydrolysis cycle, because the catalytic activities of wild-type (WT) p97 and MSP1 mutants differ when complexed with the adaptors p37 and p47.[22] p37 and p47 are trimeric proteins that contain two conserved domains: a central SEP domain that is involved in trimerization and a ubiquitin regulatory X (UBX) domain that binds to the N-domain of p97. In addition, p47 also contains an N-terminal ubiquitin-associated (UBA) domain that binds ubiquitin. Given the nucleotide-dependent conformational changes that occur in both the D1 and D2 domains of p97,[9] and the fact that nucleotide binding to one ATPase domain is coupled with ATPase activity in the other,[16,23] we sought to evaluate the effect of MSP1 disease mutations on nucleotide binding and on the binding of p37 and p47 adaptor proteins in the context of full-length (FL) p97. Results presented here provide insights into how p97's conformational changes regulate PPIs in WT and disease mutants.

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We first assessed nucleotide binding to FL WT p97 and the MSP1 mutations R155H and A232E. These two mutations were chosen because the Arg155 mutation is the most prevalent, and the A232E is associated with an earlier onset and more severe form of the disease.[17] Binding of nucleotides to MSP1 mutations was previously determined by isothermal titration calorimetry (ITC), using a construct containing only the N and D1 domains of p97.[10,16] Here, we measure nucleotide binding to FL p97 using surface plasmon resonance (SPR), which is sensitive enough to detect the binding at both the D1 and D2 ATPase sites. We have previously shown that the D1 domain binds ADP and ATP ~40-fold more tightly than the D2 domain (130 nM vs 5 μM) in the context of full-length protein.[23]

Interestingly, unlike the previous ITC binding results on the ND1 truncation of p97, which showed a decrease in ADP affinity in the D1 domain of the R155H and A232E mutants,[16] we did not observe a significant difference in the dissociation constants (K_D's) for ADP binding to either the D1 or D2 domains in FL WT or mutant p97 proteins (Table 1, Figure S1A). However, the off-rates for ADP binding were 2−3-fold faster for both the D1 and D2 domains in the R155H and A232E mutants compared to WT, indicating ADP is more easily exchanged in both domains in the mutants (Table 1). In contrast, binding of ATP and ATPγS showed similar kinetic parameters for WT and the MSP1 mutants (Figure S1B,C and Table S1).

Table 1. Binding Constants for ADP/p97 Complexes

	SPR binding constants		
	$k_a \times 10^5$ (M^{-1} s^{-1})[a]	k_d (s^{-1})[a]	K_D (nM)[b]
WT D1	6.15 ± 0.03	0.063 ± 0.001	110 ± 22
WT D2	1.85 ± 0.02	0.98 ± 0.02	5500 ± 2200
R155H D1	7.70 ± 0.09	0.141 ± 0.001	203 ± 20
R155H D2	2.58 ± 0.07	2.32 ± 0.06	10 700 ± 2000
A232E D1	18.1 ± 0.10	0.131 ± 0.001	152 ± 104
A232E D2	3.02 ± 0.09	2.66 ± 0.08	9300 ± 2400

	fluorescence anisotropy binding constants
	K_D (nM)[c]
WT D1	131 ± 11
R155H D1	185 ± 10
A232E D1	205 ± 16

[a]Errors are standard errors of the mean of the fit (duplicate data at each concentration). [b]Average K_D value calculated from kinetic fits (three independent experiments, each in duplicate). Errors are standard deviation of the mean. [c]Errors are standard errors of the mean of the fit (triplicate data at each concentration).

To confirm that immobilization of the p97 proteins did not alter the nucleotide binding potential or conformational flexibility of p97, we assessed nucleotide binding using a fluorescence anisotropy assay. We measured binding of EDA-ADP-ATTO-448 and BODIPY-FL-ATP probes to FL WT p97 and R155H and A232E mutants (Figure S2). This assay only reports binding to the D1 domain.[23] Similar to the SPR results, the K_D value for EDA-ADP-ATTO-448 binding to the D1 domain in FL WT p97 was very similar to the binding affinities to the D1 domain of the R155H and the A232E mutant (Table 1). In addition, binding affinities for BODIPY-FL-ATP to the D1 domain were within 1.6-fold for WT p97 and the MSP1 mutants (Figure S2).

To determine if MSP1 mutations altered the coupling of nucleotide binding between domains, we measured nucleotide binding to WT and R155H p97 proteins that contain Walker A mutations. Walker A mutations K251A in the D1 domain ($K251A_{D1}$) and K524A in the D2 domain ($K524A_{D2}$) prevent nucleotide binding to the respective ATPase domain. We found that the R155H−$K524A_{D2}$ mutation (where the D2 domain cannot bind the nucleotide) caused a 4-fold decrease in the affinity of ADP for the D1 domain compared to the WT-$K524A_{D2}$ mutant (Figure 1A, Table S2). This 4-fold decrease in ADP binding to the D1 domain of R155H when the D2 domain cannot bind the nucleotide is fully consistent with the ITC results using the truncated ND1 protein.[10,16] These complementary experiments underscore the importance of cross-talk for nucleotide binding in the D1 and D2 ATPase rings.[10,23] In contrast to the ADP binding results, we did not observe a difference in ATP binding to the R155H−$K524A_{D2}$ mutation compared to WT-$K524A_{D2}$ (Figure 1B, and Table S2). In addition, binding of ADP and ATP to the D2 domain ($K251A_{D1}$ mutations) was the same for WT p97 and the R155H mutant (Figure S2 and Table S2).

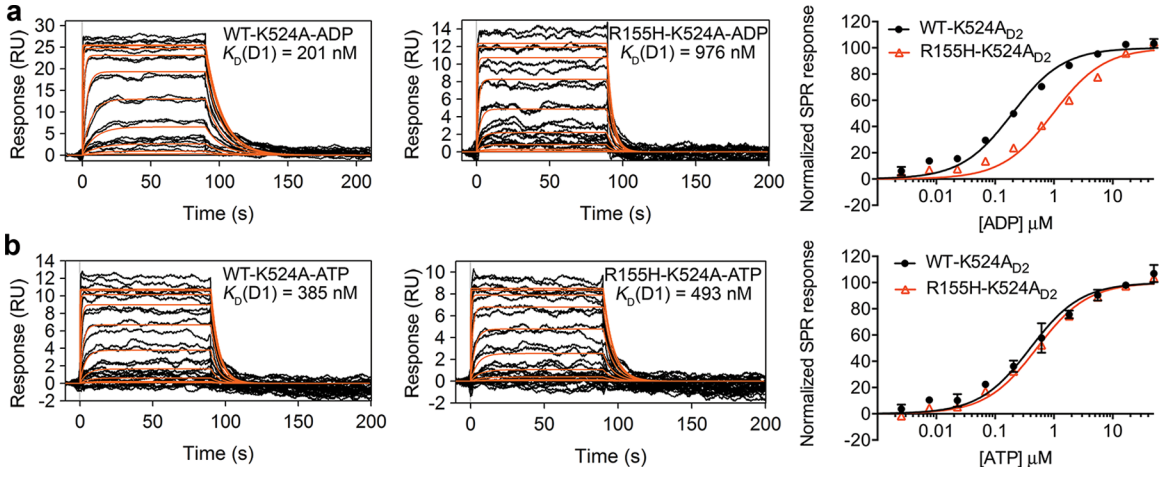

Figure 1. Representative SPR sensorgrams (black lines, n = 2) fit to a 1:1 kinetic binding model (orange lines) and normalized SPR responses taken at equilibrium fit to a one-site binding curve for (a) ADP binding and (b) ATP binding to a D2 Walker A mutation (K524A) in both WT p97 and an R155H mutant.

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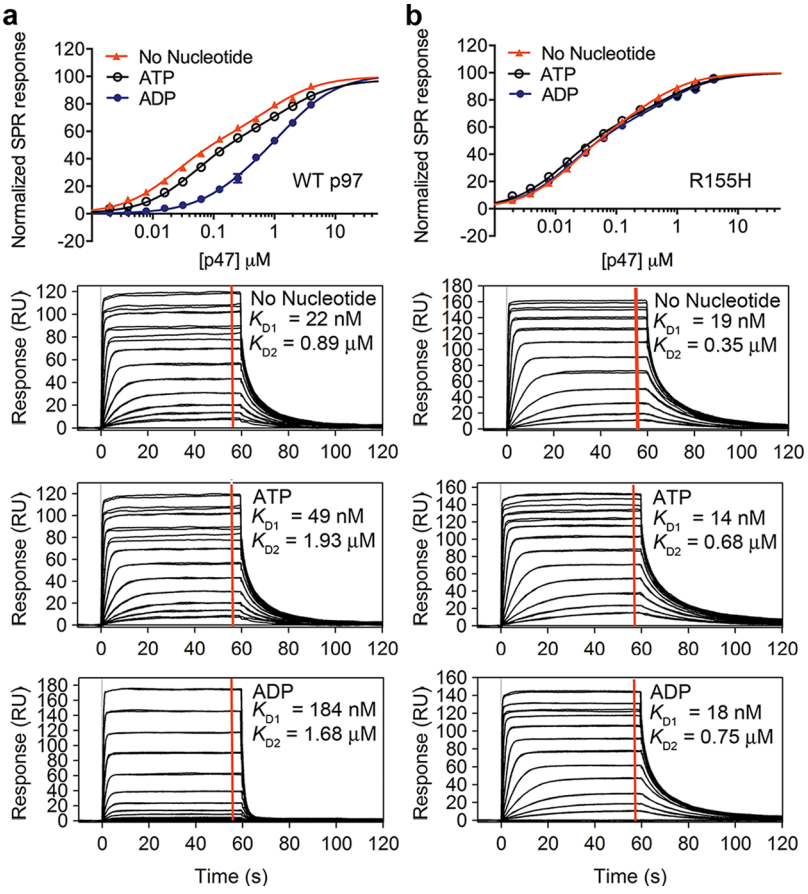

Figure 2. Binding of the adaptor p47 to p97. Normalized equilibrium binding response fit to a two-site equilibrium binding curve and representative sensorgrams for FL human p47 (0−4 μM; n = 2 at each concentration) binding to (a) WT p97 or (b) R155H in the absence or presence of 100 μM ADP or 100 μM ATP. The orange line represents the response range used to determine the equilibrium fit.

We next sought to evaluate whether the MSP1 mutations altered the conformational propensities of p97. We measured binding of the cofactors p37 and p47 in the presence of ADP (when the N-domain is in a "down" conformation, parallel to the D1 domain) and ATP (when the N-domain adopts an "up" out-of-plane conformation).[9,10,16] Because each monomer of the trimeric p37 and p47 adaptors has the potential to interact with the N-domain of p97, we hypothesized that the nucleotide-induced conformational changes in the N-domain would have an effect on adaptor binding.

We previously used SPR to measure rat p47 and human p37 binding to WT and MSP1 mutations in the absence of nucleotides.[22] We found that rat p47 bound WT p97 with two different K_D values (26 nM and 1.4 μM). Two sites could be attributed to two different binding sites within p47; a recent study suggests that the UBX domain and the SHP box (a 14-residue sequence located between the SEP and UBX domains) could bind to p97 at distinct sites on either the same or adjacent p97 monomers.[24] Other factors contributing to the two K_D values could include conformational changes/heterogeneity in p97 or the binding of p47 monomers.[22] By contrast, binding of p37 was best fit with a 1:1 binding model. However, at p37 concentrations above 256 nM, we observed nonspecific binding, which limited us from measuring a possible second binding site with micromolar affinity.

To determine if different nucleotide-bound conformations of p97 affected adaptor binding, we used SPR to measure the binding of p47 and p37 to WT and the MSP1 mutants in the absence or presence of a saturating concentration of ADP or ATP. We fit all data to a two-site model for consistency across p47/p97 measurements. When no nucleotide was added, human p47 bound to WT p97 with two K_D values (22 nM and 0.89 μM; Figure 2A), consistent with values for rat p47.[22] The binding of p47 in the presence of ATP (K_D's = 49 nM and 1.93 μM) was similar to that of apo-p97. However, when p97 was bound to ADP, the high-affinity binding for p47 was 8-fold weaker (184 nM) compared to that of apo-p97, and the off rate was significantly faster (Figure 2A). This shift was also observed for p47 binding to the ND1 domains (residues 1−458) of p97 (Figure S4), indicating that ADP binding to the D1 domain played a large role in regulating the binding of p47 to WT p97. The p37 adaptor also bound to apo-p97 and ATP-p97 with K_D values of 19 nM and 46 nM, respectively, but bound 8-fold weaker to ADP-p97 (K_D = 372 nM; Figure S5A).

In contrast to WT p97, MSP1 mutations lost their nucleotide-induced conformational selection for p47 and p37. We found that the binding affinities for p47 to the R155H mutant were similar in the absence (K_D's = 19 nM and 0.35 μM) or presence of either ATP (K_D's = 14 nM and 0.68 μM) or ADP (K_D's = 18 nM and 0.75 μM; Figure 2B). p37 also exhibited similar binding affinities to the R155H mutant regardless of the nucleotide-bound state (Figure S5B). To ensure that the loss in nucleotide-dependent conformational coupling was not specific for the R155H mutant, we also tested p47 binding to the A232E MSP1 mutant. Similarly to R155H,

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we observed tight affinity binding of p47 to the ADP-bound A232E mutant (K_D's = 21 nM and 0.71 μM; Figure S6).

On the basis of these studies, we propose a mechanistic model (Figure 3) where the p37 and p47 adaptors prefer to bind to the ATP-bound "up" conformation and may direct WT p97 into this conformation upon binding. Although a recent crystal structure of a p97 mutant in the apo-state shows the enzyme adopting an N-domain "down" conformation,[25] our results suggest that in solution nucleotide-free p97 can readily adopt the N-domain "up" conformation to promote tighter p47 binding. Additionally, nucleotide induced conformational changes likely impact the binding of other adaptor proteins; for instance, the ATP-bound form of WT p97 was also found to enhance NPL4/UFD1 binding.[26] In contrast to WT, MSP1 mutations maintain (or more readily adopt) a high-affinity conformation that we propose is the "up" conformation seen in the ATP-bound state of WT p97 (Figure 3).

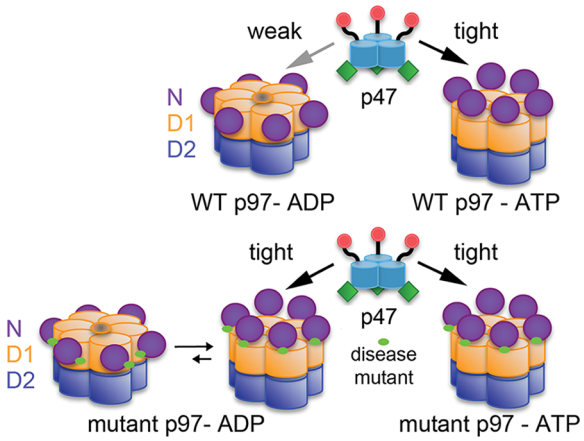

Figure 3. Model for p47 adaptor binding to WT p97 and MSP1 mutants. Cartoon illustration of a p47 trimer (UBA domain in red, SEP domain in blue, and UBX domain in green) binding to p97 (N-domain in purple, D1 domain in orange and D2 domain in blue). Light green circle indicates a MSP1 point mutation at the interface of the N and D1 domains. WT favors the N-domain "down" conformation in the presence of ADP, reducing the affinity for p47. By contrast, MSP1 mutations favor the N-domain "up" conformation and therefore bind p47 tightly even in the presence of ADP. The cofactor p37 (which lacks the UBA domain) exhibits the same conformational selection as p47.

More broadly, our results suggest that altered nucleotide-induced conformational states in MSP1 mutants may be the molecular basis for the biochemical properties observed in these pathogenic mutations. The MSP1 mutants exhibit weaker binding to ADP in the D1 domain when D2 is in a nucleotide free state (Figure 1). This altered coupling may induce more flexibility in p97's conformation as it moves through the ATPase cycle and could lead to the 3-fold higher ATPase activity observed in MSP1 mutants.[13−15] Moreover, ADP did not inhibit binding of p47 and p37 to MSP1 mutant p97 (Figure 2 and Figure S5), suggesting that MSP1 mutations may adopt the N-domain "up" conformation more easily than WT-p97 when the ADP is bound to the D1 domain. This result is consistent with the observation that MSP1 mutants bind more p47 in cells compared to WT p97[15,17,18]—MSP1 mutants bind p47 with tight affinity, independent of the nucleotide-binding state of p97. Results presented here pave the way for further studies aimed at understanding how p97 ATPase activity and conformational dynamics influence the function and stability of p97-dependent protein complexes.

METHODS

Experimental methods are detailed in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00350.

Experimental methods, Figures S1−S6, and Tables S1−S3 (PDF)

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Author Contributions

The manuscript was written through contributions of all authors.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AAA+, ATPase associated with various activities; PPI, protein−protein interaction; MSP1, multisystem proteinopathy type 1; IBMPFD/ALS, inclusion body myopathy with Paget's disease and frontotemporal dementia/amyotrophic lateral sclerosis; FL, full-length; WT, wild-type; UBX, ubiquitin regulatory X; UBA, ubiquitin-associated; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance

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